ON THE RELATION OF WHITE BLOOD CELLS AND PLATELETS TO VENOM HEMOLYSIS*

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(Received for publication, June 22, 1956)

It has been shown recently that the number of leukocytes in preparations of erythrocytes may sometimes determine whether the red cells are lysed by cobra venom (1). Thus, ox’s erythrocytes resistant to venom were rapidly lysed if the white cell count in the hemolytic system was increased to about 500/mm.³. It seemed possible that this finding might help to explain certain conflicting data in the literature and also some of the curious differences in susceptibility of red cells noted not only among species but also amongst individuals of the same species. For the leukocyte count in animal blood may run normally above 20,000/mm.³ (2) and the numbers of these cells in hemolytic systems is generally disregarded.

The nature of the component of white cells and platelets which contributes to lysis of red cells has now been examined: it appears to be a lipide. The effect on hemolysis of removing white cells and platelets as completely as possible has also been studied. Significant decreases and even absence of the hemolytic effect of venom usually occur, although there is one notable exception: the guinea pig. The findings bear upon the old problem of the mode of the direct action of a native venom, which is still unexplained (3).

Methods

Venoms were obtained as dry powders from Ross Allen’s Reptile Institute, Silver Springs, Florida. Studies were limited to specimens from Naja naja and Crotalus atrox. They were studied electrophoretically and the findings corresponded to those described, respectively, for cobras (4) and vipers (5). In barbital buffer at pH 8.6 most of the protein of Naja naja venom was basic, while most of that of Crotalus atrox was acidic. It is worth noting that the characterizations of venom phospholipases carried out by others indicate that they may differ considerably in physico-chemical behavior. Thus, crotoxin has an isoelectric point of 4.7 (6), while a lecithinase of Naja naja has an isoelectric point of 8.6 (7).

Tests for hemolysis were made with serial dilutions of venom in buffer, as previously described (1). Red cell suspensions of peripheral blood were washed and finally made up to

* This research has been aided by a grant from the United States Public Health Service (Grant C-2473).
about 1 per cent in saline. The behavior of preparations from which the white blood cells and platelets were removed as completely as possible by careful aspiration during washing of the surface buffy coat was compared with preparations in which no effort was made to exclude these cellular elements.

Consideration of the problem of analyzing leukocytes to determine the chemical nature of the material in them that contributes to venom hemolysis led at once to the difficulty of obtaining homogeneous preparations. It was decided therefore to study platelets instead, since it was reasonable to suppose that both types of cell might well supply phosphatides for the formation of lysophosphatides and thus bring about hemolysis. Ox platelets were prepared from heparinized peripheral blood by differential centrifugation in a refrigerated centrifuge. They were washed several times in saline, to a point at which microscopic examination disclosed only rare red cells. Portions of platelets were lyophilized and the lipides extracted with ethanol-ether (1:1) or chloroform-methanol (4:1). These were filtered, dried in vacuo in a rotating flask, and the residues taken up in chloroform and centrifuged or filtered to clear. The extracts were then dried and emulsified in saline and tested by addition to preparations of venom and washed guinea pig or human cells. The material insoluble in lipide solvents was tested in similar fashion.

Inasmuch as Lea, Rhodes, and Stoll (8) have recently described a lysolecithin spot in paper chromatograms of natural egg yolk, some platelet material was extracted and chromatographed according to their method. This technique, involving the use of silica-impregnated paper, proved much more satisfactory for lipide analysis than chromatography on untreated papers (9). Hack's (10) method of developing chromatograms on filter paper discs failed to give good separation of phosphatides and lysophosphatides.

Chromatography on silica paper was likewise used to test venoms for phospholipase activity. A dilute emulsion of fresh egg yolk (20 per cent V/V in buffer) was incubated for 2 hours at 45°C. with solutions of venom in varying concentrations. The material was then lyophilized, extracted with acetone, and dissolved in 20 per cent methanol in chloroform. This extract was filtered and passed through a cellulose powder column to remove amino acids (11). The chloroform eluate was collected and evaporated to such a volume that the final lipide concentration was about 5 per cent. This was subjected to descending chromatography on silica paper in 20 per cent (V/V) methanol:chloroform (8).

The papers were dried in air, sprayed on both sides with 0.2 per cent ninhydrin in n-butanol, heated at 95°C. for 5 to 10 minutes and the spots marked. After heating at 110°C. for 25 minutes the papers were finally stained with phosphomolybdic acid according to the method of Levine and Chargaff (12).

Some difficulty was met in attaining the proper degree of impregnation of papers with silicic acid. As Kirchner and Keller state (13), it is possible to wash excessively in removing chloride, and thereby to leach out too much of the silica. It has been said that the papers should be washed free from chloride (8). However, it was found that if washing was carried to the point of getting a negative test with AgNO₃ too much silica was lost and the papers were unsatisfactory.

RESULTS

Relation of Hemolysis to Numbers of White Cells and Platelets.—When the red cell preparations were largely freed of white cells and platelets, hemolysis was usually much reduced. Thus, human red cell suspensions made in the ordinary way without regard for content of other formed elements were usually lysed by cobra venom in titer 1:16,000 to 1:32,000. If the buffy coat was removed, there was regularly about a fourfold decrease in titer (Table I). On
the other hand, the titer of cobra venom against guinea pig cells did not seem to be influenced by the removal of white cells and platelets: it was generally above 1:16,000 and remained so.

Indeed, in these experiments the most convincing evidence for direct hemolytic action of venom was limited to effects on guinea pig cells. This was true for rattlesnake as well as cobra venom. In concentrations as high as 1:2,000 the venom of *Crotalus atrox* produced regularly agglutination of several species of red cells but hemolysis was seen only irregularly and incompletely except in the case of the guinea pig.

The addition of platelets greatly enhanced the hemolytic activity of venom towards all species of cells tested. The platelets were suspended in saline and added in amounts such that their final concentration approximated 100,000/mm³; i.e., about one hundred million platelets were present in the total volume of the hemolytic system. The red cells of rabbit, guinea pig, man, cat, rat, and sheep were hemolyzed rapidly by titers of cobra venom of 1:64,000 to 1:256,000. Human erythrocytes resistant to rattlesnake venom were similarly lysed upon the addition of platelets. It appeared, therefore, that hemolytic activity could be affected by the presence of platelets as well as leukocytes (1).

**TABLE I**

*Cobra Venom Hemolytic Titers* (*× 10⁴*) against Human and Guinea Pig Cells, with and without Buffy Coat Layers included in the Red Cell Suspensions

*Read after 2 hours at 37°C.*

<table>
<thead>
<tr>
<th>Guinea pig No.</th>
<th>Human No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>With buffy coat</td>
<td>16 16 — —</td>
</tr>
<tr>
<td>Without “”</td>
<td>16 16 64 64 128</td>
</tr>
</tbody>
</table>

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**Nature of the Material in Platelets Contributing to Hemolysis.**—A first approximation to the amount of phospholipide contributed by platelets added as above may be got from the analyses and calculations of Erickson *et al.* (14), who found about 0.2 mg. dried platelet material per ml. of human blood, of which some 12 per cent was phospholipide. Since this would represent roughly 3 × 10⁹ platelets, one hundred million platelets should contain rather less than 10 µg. of phospholipide. (In the present work measurements of the dry weight of platelets also indicated that one hundred million cells weigh about 0.1 mg.).

The question arises whether this amount of phospholipide if converted into lysophospholipides could be responsible for hemolysis. Wilbur and Collier (15), using a 0.5 per cent suspension of rabbit red cells, found that a lysolecithin concentration of 8 µg./ml. produced hemolysis in less than 2 minutes. It seemed entirely possible, therefore, that very small amounts of phospho-
lipide could be responsible for the increase in hemolytic activity of venom observed upon addition of white cells and/or platelets, presumably by the formation of lysophosphatides.

Consistent with this idea was the finding that the effective component of platelets was extracted completely by lipide solvents. Chromatograms of the active lipide fraction showed spots corresponding in $R_f$ values and staining to egg phosphatidylethanolamine and egg lecithin (8), as shown schematically in Fig. 1. A third, slow moving spot has already been described in natural egg yolk by Lea, Rhodes, and Stoll (8), who found it to be hemolytic and to correspond in $R_f$ value to lysolecithin prepared by treatment of yolk with venom phospholipase.

Unstained papers matching the stained strips were cut appropriately and eluted with chloroform. The eluates were evaporated to dryness, emulsified in a little saline, and tested for hemolytic activity against guinea pig cells. In natural egg yolk a small spot of lysolecithin was sometimes found. Platelet lipides appeared to contain hemolytic phospholipide also, but its $R_f$ value corresponded to lecithin rather than lysolecithin. Further experience with the method indicated that elution of silicated papers may give false positive tests.

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![Figure 1: Schematic representation of chromatogram of phospholipides from platelets and from egg yolk compared. L, synthetic lecithin L-$\alpha$-dimyristoyl (LaMotte Chemical Products Co., Baltimore); P, platelets; E, egg yolk; EV, egg yolk after incubation with venom; spots staining with ninhydrin: phosphatidyl-ethanolamine, $R_f$ about 0.8; lysophosphatidylethanolamine, $R_f$ about 0.5); --, spots staining with phosphomolybdic acid: lecithin and lysolecithin.](image-url)
for hemolysis. With the amounts of platelet material presently available it has not been possible to characterize these components further. It would appear, however, that lipide extracts of platelets contain phospholipides with solubility patterns and staining properties which closely resemble those of egg yolk lipides and are therefore likely sources of the lysophosphatides found after venom action.

Relation of Phospholipase Activity of Venom to Its Hemolytic Activity.—It has been stated that the lecithinase activity of venoms does not correlate well with their hemolytic activity (3). However, the experimental evidence offered

![Fig. 2. Schematic chromatograms of phospholipides of egg yolk before and after incubation with venom of Crotalus atrox. E, natural egg yolk; EV₁, venom dilution 1:1,000; EV₂, venom 1:10,000; EV₃, venom 1:100,000; Markings as in Fig. 1.](image)

Fig. 2. Schematic chromatograms of phospholipides of egg yolk before and after incubation with venom of Crotalus atrox. E, natural egg yolk; EV₁, venom dilution 1:1,000; EV₂, venom 1:10,000; EV₃, venom 1:100,000; Markings as in Fig. 1.

for lecithinase activity has usually been of an indirect kind, depending on an end point of hemolysis or on inhibition of the heat coagulation of egg yolk (16). It seemed worthwhile, therefore, to reexamine this question using washed guinea pig cells as the test object for direct hemolytic effects, and paper chromatography of lipide extracts of the end products of venom action on egg yolk as an indication of the extent to which natural phospholipides were converted to lysophosphatides.

At a dilution of 1:2000 Crotalus atrox venom produced partial hemolysis of guinea pig erythrocytes and failed to lyse the cells of other animal species. It appeared therefore to possess only very weak direct lytic activity.

Nonetheless, at a final concentration of 1:100,000 the same sample of
rattlesnake venom hydrolyzed the natural lecithins and cephalins of egg yolk almost completely within 2 hours. Chromatograms of the lipide extracts are shown schematically in Fig. 2.

Similar findings were encountered when cobra venom was tested in the same way. Indeed, a very basic protein could be separated electrophoretically which showed no hemolytic action whatever yet contained a potent phospholipase.

**DISCUSSION**

The findings indicate that in appraising the direct hemolytic action of venoms on certain species of erythrocytes it is necessary to take into account the presence of white blood cells and platelets. Even in the comparatively small numbers that may contaminate ordinary red cell suspensions both these cell types may enhance the hemolytic activity of venoms. They do this apparently by serving as sources of phosphatides for the formation of lysophosphatides.

On the other hand, guinea pig red cells appear to be lysed in high titer, at least by cobra venom, even though white cells and platelets are removed as completely as possible. This suggests that it might be well to use guinea pig cells as the standard test object for evaluation of the direct hemolytic action of venoms. Too often, experiments are reported which fail to give adequate biological data (20).

The development of a satisfactory method of paper chromatographic separation of phospholipides by Lea, Rhodes, and Stoll (8) makes possible a direct and convenient approach to the question whether phospholipases are indeed responsible for the direct lytic action of venoms on erythrocytes. It is clear that some venom phospholipases capable of forming lysophosphatides from egg yolk may be unable to attack red cells. On the other hand, the nature of the direct lysin in cobra venom for guinea pig cells remains unknown. It may or may not be a lipase.

In this respect it is of some interest to consider the case of crotoxin, the first snake poison to be isolated in crystalline form (17), and generally stated to embody the neurotoxic, lecithinase, and hemolytic activities of the venom. But in the communication describing the isolation of crotoxin details of the tests for hemolysis are not given. From an earlier paper in the same series (18) it appears that egg lecithin was added to the hemolytic system. Likewise, De, in studies of crystalline lecithinase of the venom of *Naja tripudians* provided a source of lecithin (19). It would appear, therefore, that substantial evidence for direct action of venom phospholipases on red cells is still wanting. The facts are perhaps most satisfactorily reconciled in the view that phospholipases bring about hemolysis if there is an extracellular source of phospholipide, including white cells and blood platelets, which can be converted into lysophospholipide, and that certain venoms may also exhibit a direct hemolytic action due to a toxin as yet unidentified (20).
SUMMARY

Removal of the white cells and platelets from suspensions of red cells usually produces substantial reduction in the hemolytic activity of venoms.

Guinea pig red cells constitute a notable exception and may be lysed by a direct action of venom.

White blood cells and platelets appear to contribute to hemolysis by serving as sources of phosphatides for the formation of lysophosphatides.

No correlation could be found between phospholipase activity and direct hemolytic activity of venoms.

A recently described method (8) of paper chromatographic separation of phospholipides has been used successfully in part of the work.

BIBLIOGRAPHY